

## Full-length article

## Fibrinogen interaction of CHO cells expressing chimeric $\alpha$ IIB/ $\alpha$ v $\beta$ 3 integrin

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### Key words

integrin; cell adhesion; fibrinogen; signal transduction

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### Abstract

**Aim:** The molecular mechanisms of the affinity regulation of  $\alpha$ v $\beta$ 3 integrin are important in tumor development, wound repairing, and angiogenesis. It has been established that the cytoplasmic domains of  $\alpha$ v $\beta$ 3 integrin play an important role in integrin-ligand affinity regulation. However, the relationship of structure-function within these domains remains unclear. **Methods:** The extracellular and transmembrane domain of  $\alpha$ IIB was fused to the  $\alpha$ v integrin cytoplasmic domain, and the chimeric  $\alpha$  subunit was coexpressed in Chinese hamster ovary (CHO) cells with the wild-type  $\beta$ 3 subunit or with 3 mutant  $\beta$ 3 sequences bearing truncations at the positions of T741, Y747, and F754, respectively. The CHO cells expressing these recombinant integrins were tested for soluble fibrinogen binding and the cell adhesion and spreading on immobilized fibrinogen. **Results:** All 4 types of integrins bound soluble fibrinogen in the absence of agonist stimulation, and only the cells expressing the chimeric  $\alpha$  subunit with the wild-type  $\beta$ 3 subunit, but not those with truncated  $\beta$ 3, could adhere to and spread on immobilized fibrinogen. **Conclusion:** The substitution  $\alpha$ IIB at the cytoplasmic domain with the  $\alpha$ v cytoplasmic sequence rendered the extracellular  $\alpha$ IIB $\beta$ 3 a constitutively activated conformation for ligands without the need of “inside-out” signals. Our results also indicated that the COOH-terminal sequence of  $\beta$ 3 might play a key role in integrin  $\alpha$ IIB/ $\alpha$ v $\beta$ 3-mediated cell adhesion and spreading on immobilized fibrinogen. The cells expressing  $\alpha$ IIB/ $\alpha$ v $\beta$ 3 have enormous potential for facilitating drug screening for antagonists either to  $\alpha$ v $\beta$ 3 intracellular interactions or to  $\alpha$ IIB $\beta$ 3 receptor functions.

### Introduction

Integrin is a family of cell adhesion molecules, which are heterodimeric transmembrane receptors composed of various  $\alpha$  and  $\beta$  subunits<sup>[1]</sup>. The primary function of integrins is the mechanical connection of cells to the extracellular matrix or to other cells by binding to specific ligands<sup>[1,2]</sup>. Ligand binding to integrins leads to the generation of intracellular signals, which in concert with other signals, coordinate cell adhesion with cell migration, growth, and differentiation<sup>[3,4]</sup>.

The  $\alpha$ v $\beta$ 3 integrin is found in many cell types and influences cell adhesion and migration with effects on angiogenesis, restenosis, tumor cell invasion, and atherosclerosis. It was

shown to be critical in angiogenesis induced by both basic fibroblast growth factor and tumor necrosis factor (TNF)<sup>[5–7]</sup>, and blocking of this integrin prevents angiogenesis in several models. Basic fibroblast growth factor and TNF costimulate  $\alpha$ v $\beta$ 3 expression on developing blood vessels in the chick chorioallantoic membrane and on the rabbit cornea<sup>[6,8]</sup>. Integrin  $\alpha$ IIB $\beta$ 3 shares the common  $\beta$ 3 subunit with  $\alpha$ v $\beta$ 3, is a receptor for fibrinogen, von Willebrand factor (vWF), fibronectin, and vitronectin (VN), and is essential for platelet aggregation<sup>[9,10]</sup>.

Integrins have been implicated in a wide variety of post-receptor occupancy events that occur as a result of ligand

binding. These include the activation of cytoplasmic protein tyrosine kinases, increased intracellular pH, and gene induction<sup>[3,11]</sup>. Additionally, intracellular signaling events can modulate integrin ligand-binding affinity, a process termed “activation” or “inside-out” signaling<sup>[4,12]</sup>. This has been best characterized in the platelet fibrinogen receptor integrin  $\alpha$ IIb $\beta$ 3<sup>[13–15]</sup>. A critical feature of the function of  $\alpha$ IIb $\beta$ 3 is that it is modulated by platelet agonists. As a consequence of platelet activation triggered by platelet agonists, such as thrombin, produced from the activated coagulation cascade upon vascular injury, integrin  $\alpha$ IIb $\beta$ 3 rapidly (<1 s) switches from a low-affinity to a high-affinity ligand-binding conformation of its ectodomains through conformational changes initiated by intracellular events referred to as affinity modulation, priming, or inside-out signaling<sup>[16–18]</sup>, which converge on the C-terminal cytoplasmic tails of the integrin subunits. However, the affinity regulation of  $\alpha$ v $\beta$ 3, the VN receptor, is not well understood<sup>[19,20]</sup>.

The integrin  $\alpha$  and  $\beta$  cytoplasmic domains plays key roles in integrin signaling<sup>[21–24]</sup>. It has been reported that the COOH-terminal Arg-Gly-Thr (RGT) sequence of  $\beta$ 3 is important for outside-in signaling; the T<sup>755</sup>NITY<sup>759</sup> sequence of  $\beta$ 3 containing an NXXY motif is critical to inside-out signaling<sup>[25,26]</sup>. However, the molecular mechanisms of integrin  $\alpha$ v $\beta$ 3 affinity regulation have been hampered by the lack of a suitable model in cultured cells convenient for adhesion assay. It has been reported that the binding of  $\alpha$ v $\beta$ 3 to VN is regulated<sup>[27,28]</sup>; however, the measurement of the alternation of the adhesion affinity of the  $\alpha$ v $\beta$ 3 expressing cells to VN has proven to be difficult.

To address the role of the  $\alpha$  and  $\beta$  cytoplasmic domains in the affinity regulation of  $\alpha$ v $\beta$ 3, we constructed a series of chimeric  $\alpha$ IIb/ $\alpha$ v and truncated  $\beta$ 3 molecules. The extracellular and transmembrane domains of the  $\alpha$ IIb subunit was fused to the cytoplasmic domain of the wild-type  $\alpha$ v subunit, and the chimeric gene was stably coexpressed in Chinese hamster ovary (CHO) cells with wild-type  $\beta$ 3 and the  $\beta$ 3 truncations at sites COOH terminal to T<sup>741</sup>, Y<sup>747</sup>, and F<sup>754</sup>, which have been shown to occur after hydrolysis by calpain<sup>[29]</sup>. These cells were used to test the cytoplasmic domains of  $\alpha$  and  $\beta$  on receptor affinity regulation and post-receptor signal transduction through binding to soluble or immobilized fibrinogen.

## Materials and methods

**Construction of chimeric  $\alpha$  integrin** The recombinant  $\alpha$ IIb/ $\alpha$ v gene, in which the  $\alpha$ IIb cytoplasmic sequence has been replaced by the corresponding  $\alpha$ v sequence, were prepared as follows: human wild-type  $\alpha$ v cDNA was cloned from

MDA-MB435 cells (a cell line isolated from the pleural effusion of a patient with breast carcinoma). The  $\alpha$ IIb extracellular and transmembrane PCR products were generated under the  $\alpha$ IIb cDNA template pcDNA3-IIb (a gift from the Shanghai Institute of Hematology, Ruijin Hospital, Shanghai, China) with primers 5'GCTCTA-GAAGATTGCCAGAGC-TTTGTGT 3' and 5'CCATCC-TCCACATGCCAGGACC 3' and  $\alpha$ v cytoplasmic PCR products with primers 5'GGCCATGTGGAG-GATGGGCTTTTTTAAAC 3' and 5'GGGGTACCTCAG-GCACTACCTGTCTTAT 3'. Using  $\alpha$ IIb extracellular and transmembrane PCR products and  $\alpha$ v cytoplasmic PCR products as templates, and with Ex *Taq* polymerase (Takara, Tokyo, Japan), dNTP Mix, and Ex *Taq* buffer in the first 3 rounds of PCR, we obtained PCR products that were then used as templates for PCR with primers 5'GCTCTAGAA-GATTGCCAGAGCTTTGTGT 3' and 5'GGGGTACCTCAG-GCACTACCTGTCTTAT 3'. The final products were digested with *Xba*I and *Kpn*I and inserted into pcDNA3.1 zeo (-)digested with the same enzymes, creating a pcDNA3.1 zeo (-) $\alpha$ IIb/C $\alpha$ v construct.

Plasmids with  $\beta$ 3 cDNA wild type and 3 kinds of plasmids with mutant  $\beta$ 3 cDNA bearing truncations at sites T<sup>741</sup>, Y<sup>747</sup>, and F<sup>754</sup> of the COOH-terminal, respectively, were kindly provided by the Shanghai Institute of Hematology, Ruijin Hospital. The mutations were confirmed by analysis of the recombinant cDNA in automated DNA sequencing analysis (Invitrogen, America).

**Cell culture and transfection** The CHO cells were grown in F12 medium supplemented with 10% fetal bovine serum, glutamine, and non-essential amino acid. Transfection was performed using Lipofectamine 2000 (Invitrogen, America). Each mutant  $\beta$ 3 cDNA was cotransfected with  $\alpha$ IIb/ $\alpha$ v at a ratio of 7:1. Forty-eight hours after transfection, the cells were collected and diluted into fresh medium containing Zeocin (Invitrogen, America) at 0.2 mg/mL. The cells were cultured with selective medium every 3 to 4 d until cell foci were clearly visible. The cell colonies were then collected and transferred into 24-well plates. The cells were cultured to subconfluence before expanding to larger plates. Stable cell lines expressing proteins were maintained in the culture in 0.1 mg/mL Zeocin.

**Flow cytometric analysis of chimeric integrin expression** The expression of chimeric integrins was monitored by flow cytometry using CD41a (BD Pharmingen, San Diego, California, America). The transfected cells were harvested using 0.5 mmol/L EDTA in phosphate-buffered saline (PBS), washed with PBS, resuspended at a density of  $1 \times 10^6$  cells/100  $\mu$ L in modified Tyrode's solution [2.5 mmol/L *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulphonic acid (HEPES),

150 mmol/L NaCl, 2.5 mmol/L KCl, 12 mmol/L NaHCO<sub>3</sub>, 5.5 mmol/L *D*-glucose, 1 mmol/L CaCl<sub>2</sub>, 1 mmol/L MgCl<sub>2</sub>, and 0.1% bovine serum albumin (BSA, pH 7.4], and incubated for 30 min at 37 °C with monoclonal antibodies specific to the extracellular domain of human  $\alpha$ IIB $\beta$ 3. Next, the cells were washed and exposed to the fluorescein-isothiocyanate (FITC)-F(ab) fragment of rabbit antimouse immunoglobulin G (Santa Cruz, CA, USA) at 37 °C for 30 min, and the intensity of fluorescence was quantified in a Coulter flow cytometer (FACSCalibur, Becton Dickinson, San Jose, CA, USA).

#### Binding of soluble fibrinogen to transfected CHO cells

The transfected CHO cells were resuspended at  $1 \times 10^6$  cells/100  $\mu$ L in modified Tyrode's solution with 15  $\mu$ g/mL Alexa Fluor 488-conjugated fibrinogen (Invitrogen, America) for 30 min at room temperature. After washing, the cells were resuspended and analyzed by flow cytometry.

#### Adhesion of CHO cell lines to immobilized fibrinogen

In total, 96-well plates were coated overnight at 4 °C with 25  $\mu$ g/mL fibrinogen in 0.5 mmol/L NaHCO<sub>3</sub> (pH 8.3). The wells then were blocked with 2% BSA-PBS at 37 °C for 2 h. Cell suspension ( $3 \times 10^4$  cells/well in F12 with 1% BSA) was added to the ligand-coated microtiter wells and incubated for 90 min at 37 °C in a CO<sub>2</sub> incubator. After 3 washes, cell spreading was examined under an inverted microscope (40 $\times$  objective lens). In the quantitative assays, 50  $\mu$ L of 0.3% *p*-nitrophenyl phosphate in 1% Triton X-100 and 50 mmol/L sodium acetate (pH 5.0) were added to 96 wells and incubated at 37 °C for 1 h. The reaction was stopped by adding 50  $\mu$ L of 1 mol/L NaOH. The results were determined by reading the optical density at a 405 nm wavelength.

**Antibodies, proteins, and reagents** Monoclonal antibodies against the integrin  $\alpha$ IIB $\beta$ 3 complex (CD41a) were purchased from BD Pharmingen (America). Alexa Fluor 488-conjugated human fibrinogen were purchased from Invitrogen (America). The integrin  $\alpha$ IIB cDNA clone in pCDNA3 and  $\beta$ 3 in the pCDM8 vector were provided by the Shanghai Institute of Hematology, Ruijin Hospital.

## Results

#### Gene expression of $\alpha$ IIB/ $\alpha$ v and $\beta$ 3 in CHO cells

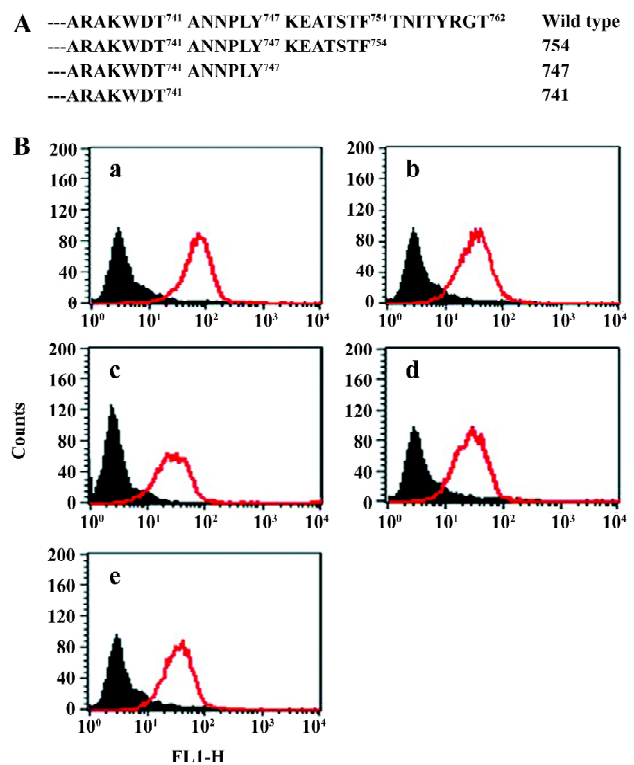
To study the structure-functional relationship of  $\beta$ 3 integrins, we generated 1 wild-type and 3 C-terminal truncated  $\beta$ 3 genes and cotransfected these genes with the chimeric  $\alpha$ IIB/ $\alpha$ v gene in Chinese hamster ovary (CHO) cells. The CHO cells cotransfected with wild-type  $\alpha$ IIB and  $\beta$ 3 genes were used as controls. Accordingly, 5 CHO cell lines were established.

In order to assess the cell surface expression of recombinant integrins transfected in CHO cells, we used flow cytometry

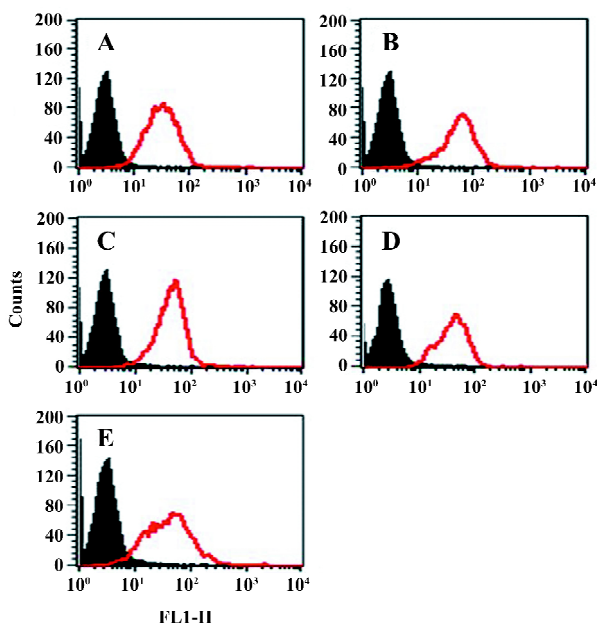
to detect the reactivity of monoclonal antibodies specific to the extracellular domain of the human  $\beta$ 3 chain (CD61). As shown in Figure 1, all the mutants used in the present study exhibited similar levels of cell surface expression of the  $\beta$ 3 subunit. Since all these cell lines express the  $\alpha$ IIB $\beta$ 3 complex in the outer side of cell surfaces, we then used a flow cytometer to evaluate their expression with the monoclonal antibodies specific to the extracellular complex of human  $\alpha$ IIB $\beta$ 3 (CD41a). As shown in Figure 2, all the cell lines expressed similar levels of the CD41a antigen, indicating similar levels of the  $\alpha$ IIB $\beta$ 3 complex on these cells.

#### Function of chimeric $\alpha$ IIB/ $\alpha$ v $\beta$ 3 integrins as a fibrinogen receptor

It is known that  $\alpha$ IIB $\beta$ 3 is a specific receptor for

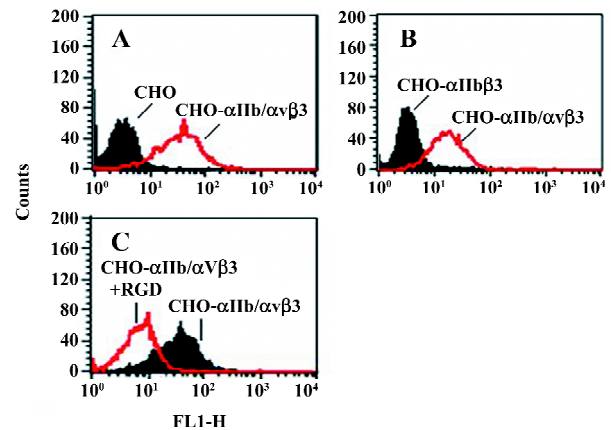


**Figure 1.**  $\beta$ 3 truncations expressed on CHO cells. (A) positions of  $\beta$ 3 truncations at the C-terminal. (B) flow cytometric analysis of the expression of extracellular  $\beta$ 3 (CD61). Control CHO cells and the transfected CHO cells were incubated with specific mouse antibodies directed against the extracellular domain of  $\beta$ 3 (CD61) for 30 min at 37 °C. After washing 3 times, the cells were incubated with FITC-conjugated goat antimouse IgG for 30 min at 37 °C. The fluorescence intensity was measured by cytometric analysis. Non-transfected CHO cells (filled histogram) were used as negative controls. (i) CHO cells transfected with  $\alpha$ IIB $\beta$ 3 genes; (ii) CHO cells with  $\alpha$ IIB/ $\alpha$ v $\beta$ 3 genes; (iii) CHO cells with  $\alpha$ IIB/ $\alpha$ v $\beta$ 3/754 genes; (iv) CHO cells with  $\alpha$ IIB/ $\alpha$ v $\beta$ 3/747 genes; (v) CHO cells with  $\alpha$ IIB/ $\alpha$ v $\beta$ 3/741 genes.

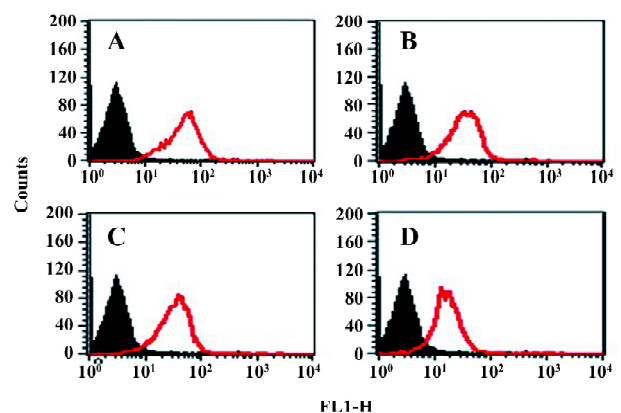


**Figure 2.** Expression of the  $\alpha$ IIb $\beta$ 3 complex (CD41a) on transfected CHO cells. Non-transfected and transfected CHO cells were incubated with specific antibodies directed against the extracellular complex of  $\alpha$ IIb $\beta$ 3 (CD41a) for 30 min at 37 °C. After washing, the cells were incubated FITC-conjugated goat anti mouse IgG for 30 min at 37 °C and analyzed with FACScan. Non-transfected CHO cells (filled histogram) were used as negative controls. (i) CHO cells transfected with  $\alpha$ IIb $\beta$ 3 genes; (ii) CHO cells with  $\alpha$ IIb/ $\alpha$ v $\beta$ 3 genes; (iii) CHO cells with  $\alpha$ IIb/ $\alpha$ v $\beta$ 3/754 genes; (iv) CHO cells with  $\alpha$ IIb/ $\alpha$ v $\beta$ 3/747 genes; (v) CHO cells with  $\alpha$ IIb/ $\alpha$ v $\beta$ 3/741 genes.

fibrinogen<sup>[9,30,31]</sup>. Thus, we first examined fibrinogen binding to the cell surface  $\alpha$ IIb $\beta$ 3 complex after the replacement of the  $\alpha$ IIb cytoplasmic domain by the  $\alpha$ v cytoplasmic domain. This was assessed by measuring the amount of fluorescence-labeled soluble fibrinogen bound to the resuspended cells without any treatment in a flow cytometer. As shown in Figure 3, the cells expressing  $\alpha$ IIb/ $\alpha$ v $\beta$ 3 bound soluble fibrinogen. This binding was inhibited by Arg-Gly-Asp-Ser (RGDS peptide), but the cells expressing wild-type  $\alpha$ IIb $\beta$ 3 could not bind soluble fibrinogen. Thus, unlike natural  $\alpha$ IIb $\beta$ 3 integrin, activation was not required for the cells bearing chimeric genes to bind soluble fibrinogen. This also indicated that the cells bearing the chimeric  $\alpha$ IIb/ $\alpha$ v gene formed the  $\alpha$ IIb $\beta$ 3 complex in a correct conformation on the cell surfaces, because RGD-dependent fibrinogen binding is a restricted functional marker highly specific for  $\alpha$ IIb $\beta$ 3<sup>[32,33]</sup>. Then we examined the soluble fibrinogen binding of the cells with the C-terminal truncated  $\beta$ 3 at the intracellular compartment. As demonstrated in Figure 4, soluble fibrinogen bound to  $\alpha$ IIb/ $\alpha$ v $\beta$ 3/741,  $\beta$ 3/747, and  $\beta$ 3/754 cells at com-



**Figure 3.** Inhibition by RGDS of soluble fibrinogen binding to the CHO cells expressing  $\alpha$ IIb/ $\alpha$ v $\beta$ 3 genes. Cells were incubated with 15  $\mu$ g/mL Alexa Fluor 488-conjugated fibrinogen for 30 min at room temperature. After washing 3 times, the fluorescence was measured in the flow cytometry. (i) non-transfected CHO cells (filled histogram) and the CHO cells expressing heterodimers of  $\alpha$ IIb/ $\alpha$ v $\beta$ 3 (open histogram); (ii) CHO cells expressing heterodimers of  $\alpha$ IIb/ $\alpha$ v $\beta$ 3 (filled histogram) and expressing  $\alpha$ IIb/ $\alpha$ v $\beta$ 3 (open histogram); (iii) CHO cells expressing heterodimers of  $\alpha$ IIb/ $\alpha$ v $\beta$ 3 after incubation with Alexa Fluor 488-conjugated fibrinogen in the presence (open histogram) or absence (filled histogram) of 2 mmol/L RGDS.



**Figure 4.** Binding of soluble fibrinogen to the CHO cells expressing truncated  $\beta$ 3. Cells were incubated with 15  $\mu$ g/mL Alexa Fluor 488-conjugated fibrinogen for 30 min at room temperature. After washing 3 times, the cell fluorescence was determined by flow cytometric analysis. CHO cells expressing  $\alpha$ IIb $\beta$ 3 as the control (filled histogram). (i) CHO cells expressing  $\alpha$ IIb/ $\alpha$ v $\beta$ 3; (ii) CHO cells expressing  $\alpha$ IIb/ $\alpha$ v $\beta$ 3/754; (iii) CHO cells expressing  $\alpha$ IIb/ $\alpha$ v $\beta$ 3/747; (iv) CHO cells expressing  $\alpha$ IIb/ $\alpha$ v $\beta$ 3/741.

parably high levels as to  $\alpha$ IIb/ $\alpha$ v $\beta$ 3/762 (WT) cells, showing that  $\alpha$ IIb/ $\alpha$ v $\beta$ 3 was in a constant activation state regardless of the truncation of  $\beta$ 3 within the intracellular tail.

**Integrins mediate cell adhesion and spreading on the**

**fibrinogen matrix** Another important function of  $\beta 3$  integrins is cell adhesion to the extracellular matrix. The cytoplasmic chain of  $\beta 3$ , especially its C-terminal, has been proven to play an important role in integrin signaling and to regulate its function. We further examined the behavior of these cell lines in adhesion and spreading on the fibrinogen matrix.

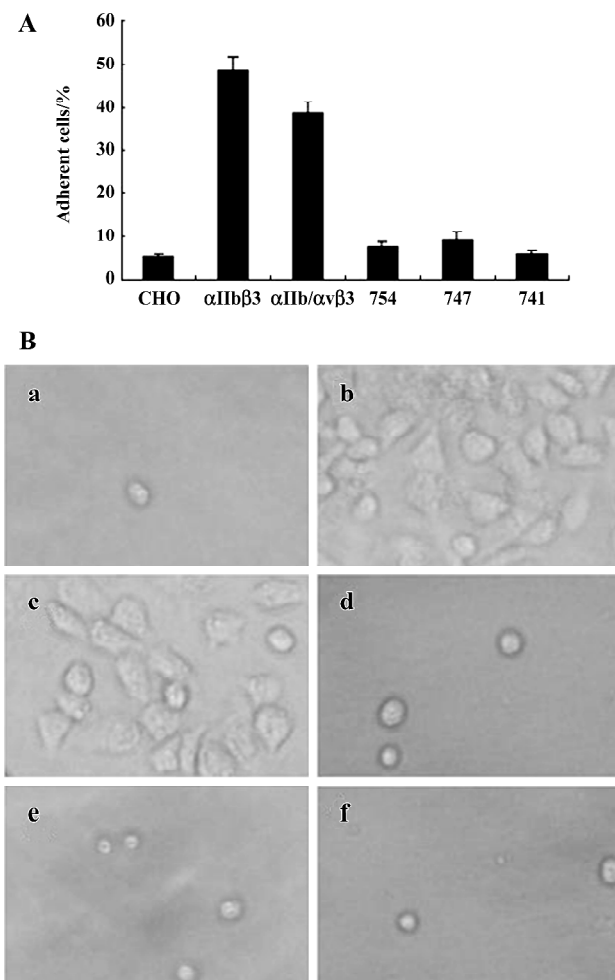
The adhesive properties of the cells expressing  $\alpha \text{IIb}/\alpha \text{v}\beta 3$  containing full-length or truncated  $\beta 3$  subunit proteins were studied. The cell lines  $\beta 3/741$ ,  $\beta 3/747$ ,  $\beta 3/754$ , and  $\beta 3/762$ , the

cell line  $\alpha \text{IIb}\beta 3$ , as well as the non-transfected control cell lines, were added to fibrinogen-coated polystyrene wells. As shown in Figure 5, non-transfected CHO cells lacking  $\alpha \text{IIb}/\alpha \text{v}\beta 3$  were incapable of adhering to the fibrinogen matrix. CHO cells expressing  $\alpha \text{IIb}\beta 3$  and the cells expressing  $\alpha \text{IIb}/\alpha \text{v}\beta 3/762$  (WT) adhered to and spread well on the fibrinogen matrix. In contrast, all CHO cells displaying truncated  $\beta 3$  in the  $\alpha \text{IIb}/\alpha \text{v}\beta 3$  complex, including  $\beta 3/741$ ,  $\beta 3/747$ , and  $\beta 3/754$ , failed to adhere to the fibrinogen matrix. These results showed that the full length of  $\beta 3$  was required for the cell adhesion to immobilize fibrinogen; any deletion of the C-terminal sequence of  $\beta 3$  would abolish  $\alpha \text{IIb}/\alpha \text{v}\beta 3$ -mediated adhesion and spreading.

## Discussion

In this study, we generated the chimeric  $\alpha \text{IIb}/\alpha \text{v}\beta 3$  integrin protein, which fused the extracellular and transmembrane sequences of  $\alpha \text{IIb}$  to the intracellular sequences of  $\alpha \text{v}$  and successfully expressed this chimeric integrin on the surface of CHO cells. This model was designed to test whether such a chimeric molecule is functionally active and if any signal transduction can be observed by the ligand binding assay and cell adhesion assay. Since  $\alpha \text{IIb}\beta 3$  binding to fibrinogen is extremely well documented and easy to test, this chimeric integrin was expected to provide a useful model for the study of signal transduction mediated by the  $\alpha \text{v}\beta 3$  intracellular domains.

The activation of  $\alpha \text{IIb}\beta 3$  has been well documented<sup>[12-14]</sup>. The natural  $\alpha \text{IIb}\beta 3$  on the platelets was inactive and turns into an active form once the cells are stimulated by agonists, such as thrombin, through inside-out signaling<sup>[30,34,35]</sup>. Our results are in agreement with this notion since fibrinogen did not bind to the CHO cells expressing  $\alpha \text{IIb}\beta 3$ , while these cells expressed high levels of the  $\beta 3$  chain (CD61) and  $\alpha \text{IIb}\beta 3$  complex (CD41a). There are also some reports about the constitutive activation of  $\alpha \text{IIb}\beta 3$  by demonstrating that the chimeric  $\alpha$  subunits with the extracellular and transmembrane of  $\alpha \text{IIb}$  joined to the cytoplasmic domains of  $\alpha 5$ ,  $\alpha 6A$ , or  $\alpha 6B$  conferred a high affinity state to the recombinant integrins<sup>[36]</sup>. In the present study, we showed that once the  $\alpha \text{IIb}$  cytoplasmic tail was substituted by the  $\alpha \text{v}$  tail, the extracellular  $\alpha \text{IIb}\beta 3$  receptors were constitutively active for their ligands in the absence of the inside-out signals. The activated state of the receptors remained unimpaired with the truncations of the cytoplasmic sequences of  $\beta 3$ , as we could see the significant amount of the soluble fibrinogen bound to the cells expressing these truncated versions of integrin  $\beta 3$ . This binding was specifically mediated by  $\alpha \text{IIb}/$



**Figure 5.** Cell adhesion and spreading on immobilized fibrinogen. (A) histogram of cell adhesion and spreading on fibrinogen-coated matrix. Indicated cell lines in F12 ( $3 \times 10^4$  cells/well) were incubated in fibrinogen-coated microtiter wells at 37 °C for 1.5 h. Wells were washed 3 times and the cells remaining adherent were quantified using an acid phosphatase assay. (B) photographed cells on fibrinogen-coated plates. Cells were photographed before the acid phosphatase assay. (i) non-transfected CHO cells; (ii) CHO cells expressing  $\alpha \text{IIb}\beta 3$ ; (iii) CHO cells expressing  $\alpha \text{IIb}/\alpha \text{v}\beta 3$ ; (iv) CHO cells expressing  $\alpha \text{IIb}/\alpha \text{v}\beta 3/754$ ; (v) CHO cells expressing  $\alpha \text{IIb}/\alpha \text{v}\beta 3/747$ ; (vi) CHO cells expressing  $\alpha \text{IIb}/\alpha \text{v}\beta 3/741$ .

$\alpha v\beta 3$  because it was completely inhibited by the RGD peptide. Based upon our data and those of others, we propose a model for integrin activation, in which the  $\alpha IIb$  and  $\beta 3$  chains within extracellular domains tend to expose fibrinogen-binding sites. This tendency is however blocked by the interaction of the intracellular part of the  $\alpha IIb$  and  $\beta 3$  subunits. Accordingly, any structural alternation that impairs the interaction of the  $\alpha IIb$  and  $\beta 3$  subunits within the intracellular domains will activate the integrin. We believe that the proposal of this model would be beneficial in understanding the mechanisms of integrin activation which is thought to be regulated by inside-out signaling<sup>[12–14]</sup>. It is tempting to speculate that the disturbance of the interaction between the intracellular and transmembrane domains of the  $\alpha IIb$  and  $\beta 3$  subunits, in the case of the chimeric  $\alpha$  subunit as in this study or of talin interaction, causes a “deblockade” of the suppressed receptor activity and this will be responsible for integrin activation<sup>[37,38]</sup>.

We showed that both the cells expressing  $\alpha IIb\beta 3$  and the cells expressing chimeric  $\alpha IIb/\alpha v\beta 3$  molecules adhered to and spread well on immobilized fibrinogen in a similar manner. In contrast, the 3 cell lines expressing truncated  $\beta 3$  at the C-terminal failed to adhere firmly and spread. Our data showed that the truncations of  $\beta 3$  did not alter the soluble fibrinogen binding capacity of  $\alpha IIb/\alpha v\beta 3$ , but it did alter the cell adhesion on immobilized fibrinogen, indicating that fibrinogen binding capacity is not sufficient to support fibrinogen-mediated cell adhesion and spreading. In fact, it showed that the cell spreading on immobilized fibrinogen was a more complicated phenomenon and depended on the integrity of intracellular  $\beta 3$ , which has been reported to play a key role in signal transduction<sup>[4,25,26]</sup>. It is known that binding of ligands to  $\alpha IIb\beta 3$  not only forms adhesive bonds between platelets, but also transmits outside-in signals to induce a series of cellular responses, such as protein phosphorylation<sup>[39,40]</sup>, elevation of intracellular  $Ca^{2+}$ <sup>[41]</sup>, and cytoskeleton reorganization<sup>[42]</sup>, leading to cell spreading and the stabilization of cell adhesion<sup>[43–45]</sup>. In particular, the hydrolysis of short peptides at the C-terminal of the  $\beta 3$  chain has been shown to be involved in this signal transmission<sup>[25,29]</sup>. Therefore, the failure of the cells with truncated  $\beta 3$  to adhere to and spread on immobilized fibrinogen could be due to the disruption of the outside-in signals that occurred in natural  $\alpha IIb\beta 3$ . This interpretation underlines once again the importance of the regulatory role of  $\beta 3$  within the intracellular  $\alpha v\beta 3$  complex in outside-in signal transmission. However, further study is needed to elucidate the detailed mechanisms.

Our data indicate that the intracellular interactions within

the  $\alpha v\beta 3$  cytoplasmic tail of the cell line CHO  $\alpha IIb/\alpha v\beta 3$  regulates the adhesion function of the extracellular  $\alpha IIb\beta 3$  domain to immobilize fibrinogen and consequent spreading. Therefore, the cell line CHO  $\alpha IIb/\alpha v\beta 3$  will be a useful model for studying the intracellular protein-protein interaction in which  $\alpha v\beta 3$  intracellular domains are involved. Furthermore, this model is potentially useful for the drug screening of active substances interfering in  $\alpha v\beta 3$ -mediated signaling. In addition, as we showed, the chimeric integrin  $\alpha IIb/\alpha v\beta 3$  was constitutively activated for ligand binding with no need of agonist stimulation, which suggests that the cell model would be also a useful tool in drug screening of new substances interfering with fibrinogen binding to  $\alpha IIb\beta 3$ , a critical step during platelet aggregation and blood clot formation.

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